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BACK

THE HYBRIDIZATION OF *CORDYCEPS SINENSIS* STRAINS AND THE MODIFICATIONS OF THEIR CULTURE PARAMETERS, IN ORDER TO OPTIMIZE THE PRODUCTION OF TARGET MEDICINAL COMPOUNDS

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ABSTRACT:

The mushroom species *Cordyceps sinensis* has long been used in folk medicine throughout the Orient. It has only been in the last few years however that Science has had the ability to thoroughly analyze this mushroom, and identify the bioactive compounds present. In the course of our research with cultivated *Cordyceps* strains over the last five years, we have noted that there is perhaps a greater biodiversity of compounds within different strains of this single species, than in almost any other organism we have analyzed. Due to the great difference in the concentration of native compounds, a wide range of quality is found in *Cordyceps* cultivated from different strains and utilizing different culture methodology. This article looks at the techniques and methods we have used in the development of hybridized strains of *Cordyceps sinensis*, and the modifications of their culture parameters such as light, oxygen concentration, temperature, substrate and mineral composition, with the goal of producing artificially cultivated *Cordyceps* for the health supplement trade, which contains the maximum content of scientifically recognized bioactive compounds. The techniques and methods detailed in this article offer great promise in allowing cultivators of *Cordyceps sinensis* and other *Cordyceps* species to take their artificially cultivated products to a higher and more consistent level of quality**.**

KEYWORDS:

Cordyceps, Snake venom, HPLC, GC, HEAA, Cordycepin, adenosine, Hydroxyethyladenosine, deoxynucleosides, fungal hybridization, culture parameter modification,HIV,anti-aging,herbal supplement,mycelium

ART I – BACKGROUND AND QUALITY ANALYSIS OF *CORDYCEPS*

BIOLOGICAL DESCRIPTION OF *CORDYCEPS SINENSIS*:

Cordyceps sinensis is an insect parasitizing fungus of the ascomycetes family, found at high altitudes on the Qinghai-Tibetan plateau. This fungus lives primarily in the larvae of one particular species of moth, *Hepialus armoricanus*. It is occasionally found growing on other moth species as well. The normal range of this fungus is above 2000 meters elevation, and it has been found as high as 6000 meters. There are also many other species of the genus *Cordyceps*, which all seem to have potent biologically active compounds present. The genus has been shown to produce some potent antibiotics, immune stimulants and antitumor agents. (Zhou, Halpern and Jones, 1998 [1]; Hobbs, 1986)

There is debate among many scientists at present whether the species of the genus *Cordyceps* are in fact single organisms or if they are symbiotic colonies of more than one organism. Perhaps what we are calling *Cordyceps sinensis* today, will one day be known as a fungal/bacterial symbiosis. DNA sequencing has proven inconclusive in this regard as the DNA sequence tends to change with time, as if the fungus were incorporating some of the insect DNA into its own DNA code for the initiation of its fruitbody form, then losing the insect DNA when it goes back into its mycelial form. Microscopic examination of growing *Cordyceps* mycelium reveals some very interesting morphology, including the concurrent anamorphs of filamentous mycelium and rapidly moving single celled yeast-like morphological forms. This has been seen in other *Cordyceps* species as well, such as *Cordyceps sobolifera*. Many times we have observed what appears to be an inner-cellular symbionts, a small, spherical, rapidly moving, apparently single celled organism living within the long, bamboo-like hyphal strands. When we observe this growth form, these secondary cells occur in about 70-80% of the mycelial cells observed, with anywhere from one to five individually moving spheres per mycelial cell. These are easily observed in the living culture under 1000-power magnification with normal back lighting.

HISTORICAL USAGE OF *CORDYCEPS SINENSIS:*

Cordyceps sinensis, known to the Chinese as “DongChongXiaCao” and to the Japanese as “Tochukaso” has been used in medicine for a very long time. The first known written record of this herbal medicine was in the Ben-Cao-Cong-Xin (*New Compilation of Materia Medica*) by the author Wu-Yiluo. Written around the year 1757 AD during the Qing Dynasty, this early medical text lists the traditional usage of *Cordyceps* as going to the Lung and Kidney meridian and being useful as a “Lung Protectorate”, for “Kidney Improvement” and as a “Yin/Yang double invigorant”. *Cordyceps* in Traditional Chinese Medicine (TCM) was and usually still is prepared by cooking the whole caterpillar/fruitbody combination in chicken or duck soup. It has been used this way for the treatment of many conditions, such as respiratory diseases, renal dysfunction, hyperlipidemia and

hyperglycemia.(Zhou, Halpern and Jones, 1998 [2])

This would seem to be a lot of medicinal claims for a single substance. But the observation of many generations of medical practitioners has lead to this belief. As people tend to be very good observers of nature, and especially when the observations are proven over a long period of time, as is the case with *Cordyceps*, there was sufficient background for further research into this herbal substance for use in modern medicine.

With many long years of successful use, modern science has been looking into this natural medicine since the 1950s.

MODERN CULTIVATION OF *CORDYCEPS SINENSIS*:

Since native *Cordyceps* (wild *Cordyceps sinensis*) is rare and very expensive, there has been a lot of research into methods for cultivation of this fungus. The strain that is known as CS-4 was one of the first commercial strains of *Cordyceps* isolated in 1982 at the Institute of Materia Medica, Chinese Academy of Medical Sciences. Known by the Latin name of *Paecilomyces hepiali* Chen, the aseptically fermented mycelium of this strain underwent extensive human testing and clinical trials during the 1980's and resulted in a commercial product with wide usage in China, known as JinShuiBao capsules. More than 2000 patients were involved in the clinical trials with CS-4 and the chemical composition, therapeutic activity and toxicity are very well known for this strain. (Bau, 1995)

A number of other strains have been isolated from wild *Cordyceps* since then. These are each so different from the original starting *Cordyceps* and from each other that they are given many different Latin Genus/species names. Even though the parent fungus is the same in each case, the resultant asexual mycelial growth forms are characteristically different enough in taxonomy and chemistry that they are considered different species by many taxonomists. Table 1 lists some of the different strains isolated from wild *Cordyceps sinensis*. (Yin and Tang 1995; Zhao, Wang, Chen, Li and Qu, 1999)

Table 1

Latin binomial	Isolated by	Commercial product
Cephalosporium sinensis	QingHai Institute of Livestock and Vetrinary sciences	NingXinBao
Paecilomyces sinensis Cn80-2	FuJianQingLiu County Hospital	various
Scydalilum sp.	Sanming Mycological Institute	SM1H8819
Scydalilum sp.	Chinese Navy Institute of Medicine	832
Hirsutella sinensis		various
Mortierella hepiali Chen Lu		various
Topycladium sinensis		various
Scytalidium hepiali G.L.Li		various

ADULTERATED AND COUNTERFEIT *CORDYCEPS SINENSIS*:

With the opening of China to business with Western countries in the

1970s, many people in countries far from China were exposed to the benefits found in TCM. Along with this exposure to the traditional medical methods came a great demand for the herbal medicines used in that medical system. The great demand worldwide for *Cordyceps*, and the enormous cost of the wild collected variety has led to many unscrupulous manufacturers and distributors providing adulterated and counterfeit *Cordyceps* in the world market. (Hsu, Shiao, Hsiea and Chang 2002) Most of the Western world prefers their medicine to come in clean white bottles and neat little capsules, rather than in the whole caterpillar form. This makes it even easier and more tempting for some suppliers to sell just about anything under the label of "*Cordyceps*". In an attempt to identify what "real" *Cordyceps* was, we started analyzing all of the available *Cordyceps*, both commercial products and bulk raw material products, grown by nearly all of the cultivators and suppliers worldwide. What we found was shocking. Nearly all of the commercially available *Cordyceps* products available in the United States that were imported from China, contained no detectable amounts of *Cordyceps* whatsoever. The results of testing on American produced *Cordyceps* were a little better. In every case with American *Cordyceps* we were able to recognize the characteristic analytical signature of *Cordyceps*, but in none of the American samples was there any significant amount of active ingredients. The American grown *Cordyceps* products consisted almost entirely of unconverted grain substrate upon which the *Cordyceps* is grown.

TEST METHODS AND STANDARDS OF QUALITY:

The methods for analyzing *Cordyceps* quality have not yet become standardized throughout the world. Every lab that is conducting this type of testing uses their own methods and their own standards. So when we first began our analysis of *Cordyceps* in 1999, we had to develop our own test methods. We tried many different test protocols before settling on the following two, as being accurate, repeatable and relatively economical.

GAS CHROMATOGRAPHY WITH MASS SPEC DETECTION (GC/MS):

Trimethylsilyl Derivative Method: Starting with well dried and finely ground powder of the raw test sample, 20 mg is added to 0.3 ml of derivatizing agent (BSTFA) and 0.3 ml of acetonitrile. This mixture is heated for 20 minutes at 60 degrees C, which yields a Trimethylsilyl derivative, which carries the material through the GC for detection with a mass spectrometry detector. This test method is simple and quick, and it yields a yes or no answer as to whether the test sample is actually *Cordyceps* or not. This method can be used for quantification of the target compounds, although the next method is more accurate and more suitable for complete target compound quantification:

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY / MASS SPEC DETECTION:

Powdered samples (2.0g) were defatted by decanting with hexane (3 x 50ml) and dried in vaccuo. Samples were dissolved in 0.1M TBE

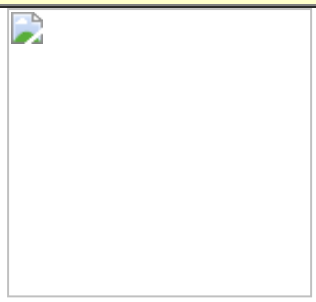
(Tris-borate-EDTA) buffer (pH 8.5 with 0.1N NH_4OH) (100ml) and sonicated for 30 minutes at 40°C. An aliquot (10ml) of the sample was then passed through a C-18 Sep-Pak that had previously been pre equilibrated with 0.1M TBE (Tris-borate-EDTA) buffer (pH 8.5 with 0.1N NH_4OH). The eluent was collected, and the Sep-Pak further washed with the equilibration buffer to give a final eluent volume of 20 ml. After thoroughly mixing the sample was filtered through a 0.45 micron PVDF membrane and placed into suitable vials for HPLC-MS analysis. The chromatography was performed on a Waters 2695 separation module using a Wako Wakosil-II 5C18 HG column (5 mum, 15 cm * 4.6 mm i.d.) at 45°C with gradient elution of H_2O:methanol (1 ml/min) from 22:3 to 77:23 in 19 min, then to 18:7 at 24 min and 27:23 at 39 min. The chromatographic eluent was passed into a Vestec particle-beam interface for solvent removal and particle atomization and then via Teflon transfer line into the mass spectrometer using a helium carrier gas. Detection was performed on a Finnigan TSQ7000 triple-quadrapole mass-spectrometer in positive ion mode with full scan centroid data collection (50-1000 m/z). MS-MS experiments using an argon collision gas were used to verify the identity of unusual nucleotides for which no primary standards were available.

STANDARDS OF QUALITY:

Quality determination in *Cordyceps* for the Health Supplement industry has been haphazard up until now, as there have been no universally recognized test methods for analyzing this particular supplement. Each company producing or supplying *Cordyceps* has used different tests, or tested for different substances in order to show their product standing out above all the rest. Some analyze for Adenosine, some for Cordycepin, some for Cordycepic Acid, and some for particular sugars or total polysaccharides. While all of these tests have some usefulness in determining relative quality, none of these tests by themselves are in any way meaningful when it comes to whether the product in question will yield good results when used as medicine for humans or not. Almost all of the samples of wild collected *Cordyceps* analyzed very similar in chemical composition, but we quickly realized that there was a tremendous variation in the secondary metabolite compounds present in cultivated *Cordyceps sinensis* and other *Cordyceps* species. A greater variation in compounds than we had found in any other organism. So our first step was to determine which compounds we could use as targets to equate with medicinal potency. After a thorough review of the literature, the nucleosides, and specifically the deoxy-nucleosides were determined to be the most reliable indicators of potency. This class of compounds has been carefully studied and reported on in the scientific literature, and was the class of compounds showing the most variation in different samples of cultivated *Cordyceps*. Furthermore, many of the deoxy-nucleosides are found in no other organism, or at best, a very limited number. We chose the compound N^6-(2-hydroxyethyl)-Adenosine as our indicator compound, since we found this in all specimens of *Cordyceps* and have not found it in any other organism. This compound, along with Adenosine and

3’deoxyadenosine (Cordycepin) were used in summation as the quality indicator to compare different strains and production methods of *Cordyceps*. That is, the quantities of the three compounds were added together to come up with a numerical quality index for *Cordyceps*. (Furuya, Hirotani and Matsuzawa, 1983) Structures for these three compounds are shown in Table 2:

Table 2

		
N^6-(2-hydroxyethyl)-Adenosine	**Cordycepin (3’deoxyadenosine)**	**Adenosine**

There are a number of other interesting deoxy-nucleosides produced by *Cordyceps sinensis*, such as the compound 2’,3’ deoxyadenosine which is marketed in the United States

as a drug for the treatment of AIDS under the trade name “Didanosine”. There are also several varieties of deoxy-uridines present, but as there is not much literature on these compounds yet, we decided not to use these as quality index indicators. In time we will learn what effect these unique compounds have in the human body and may find that these compounds are as important a group as the adenosine-type compounds, which we refer to in this paper as HEAA (**H**ydroxy **E**thyl **A**denosine **A**nalogs). We find that the HEAA content is a much more reliable indicator of *Cordyceps* quality than could be determined through testing of the polysaccharides. The reason for this has to do with the method of analysis for polysaccharides, which is usually done by wet-chemistry methods, by breaking the polysaccharide bonds through acid hydrolysis or enzymatic activity, and measuring the quantity of simple sugars present after cleavage. We find this method unreliable as a quality indicator, since the residual sugars tell nothing of their source or their linkage characteristics (ie: alpha bonded or beta bonded), and the test results are easily altered through the addition of starch or other polysaccharides to the raw material at the manufacturing stage. Rather, by using this HPLC/MS method, any sample of *Cordyceps* from any source is quickly and easily analyzed and a numerical quantity index can be applied to it. When this method becomes universally applied in the health supplement industry, producers will be forced to address the quality issue of their products, and the industry as a whole will benefit. There will be much less adulterated or counterfeit product placed on the market and consumer confidence will grow as a result. Most consumers of wild *Cordyceps* already know that it is normal practice for collectors to insert small segments of twigs or even pieces of wire into the body of the caterpillars to increase the weight. Many consumers of capsulated *Cordyceps* do not know what

real *Cordyceps* even tastes or smells like. We have analyzed some specimens of "*Cordyceps* Capsules" which contained nothing but rice flour, and other samples which contained nothing but nutmeg. It is hoped that by the application of the test methods given here, such deceptive production practices will stop.

ANALYTICAL RESULTS:

We conducted analysis over the course of 4 years on samples from what we believe to be every commercial producer of cultivated *Cordyceps* in the world. The results obtained through testing these approximately 100 samples from producers in 4 countries is shown in table 3:

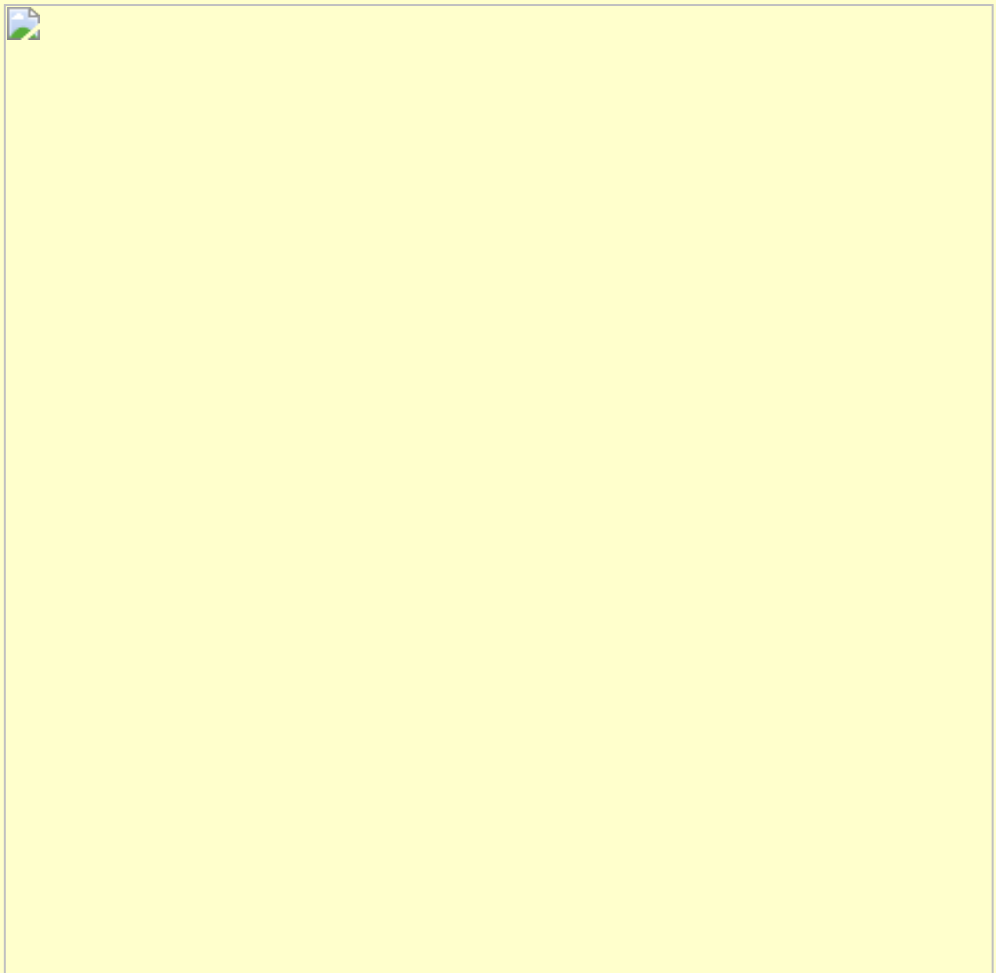
Table 3

	American mycelium	Oriental mycelium	Standardized Extracts	Wild *Cordyceps*	Aloha Medicinals hybrid *Cordyceps*
HEAA Range	0.00%-0.41%	0.00%-0.071%	0.050%-0.073%	0.071%-0.10%	0.190%-0.323%
HEAA Average	0.005%	0.025%	0.070%	0.09%	0.303%

NOTE: Test results through the HPLC/MS methods listed here.

It can easily be seen from Table 3 that there is quite a difference in quality of *Cordyceps* from different producers. These quality differences become even more pronounced when seen in graphic format. Plot 1 below is a typical HPLC plot of wild collected *Cordyceps*:

PLOT 1 – WILD *CORDYCEPS*



TOTAL HEAA CONTENT IN THIS SAMPLE IS about .091% w/w

NOTE: This is the basic signature of *Cordyceps sinensis* that we use for comparative purposes when determining whether a sample is actually *Cordyceps* or not.The next plot is a typical American grown CS-4 strain *Cordyceps* grown on a solid substrate of grain. Actually, most grain-grown American mycelium shows less active ingredients than this sample. This can be considered as "Good Quality American *Cordyceps*". The bioactive ingredient quantities are much lower than the wild *Cordyceps*, and not all of the secondary metabolite compounds are present in this sample, due to the culture methods, substrate and strain. See Plot 2:

PLOT 2 – AMERICAN CS-4 *CORDYCEPS* GROWN ON GRAIN

TOTAL HEAA CONTENT IN THIS SAMPLE IS about .006% w/w

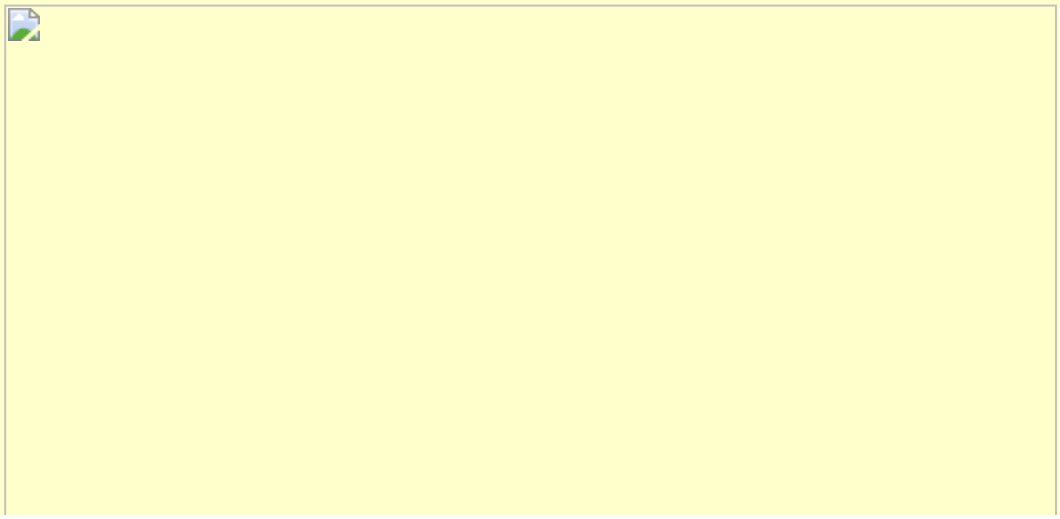
Plot 3 shows a typical liquid cultured (fermented) powdered extract of *Cordyceps* mycelium produced in China. This is the best quality *Cordyceps* mycelium product we ever tested other than the Aloha Medicinals Inc. hybrid *Cordyceps* sinensis, which is detailed in the next section of this paper. See Plot 3:

PLOT 3 – CHINESE GROWN *CORDYCEPS SINENSIS* 4:1 EXTRACT

TOTAL HEAA CONTENT IN THIS SAMPLE IS about .073% w/w

Plot 4 is a raw HPLC plot of an American grown "*Cordyceps militaris*" This plot is included here to show what we sometimes find in analyzing *Cordyceps* samples. It can be seen from this plot that this sample contains none of the characteristic compounds found in *Cordyceps*. Whatever this powder was, it was not *Cordyceps*. See Plot 4:

PLOT 4– *CORDYCEPS MILITARIS*

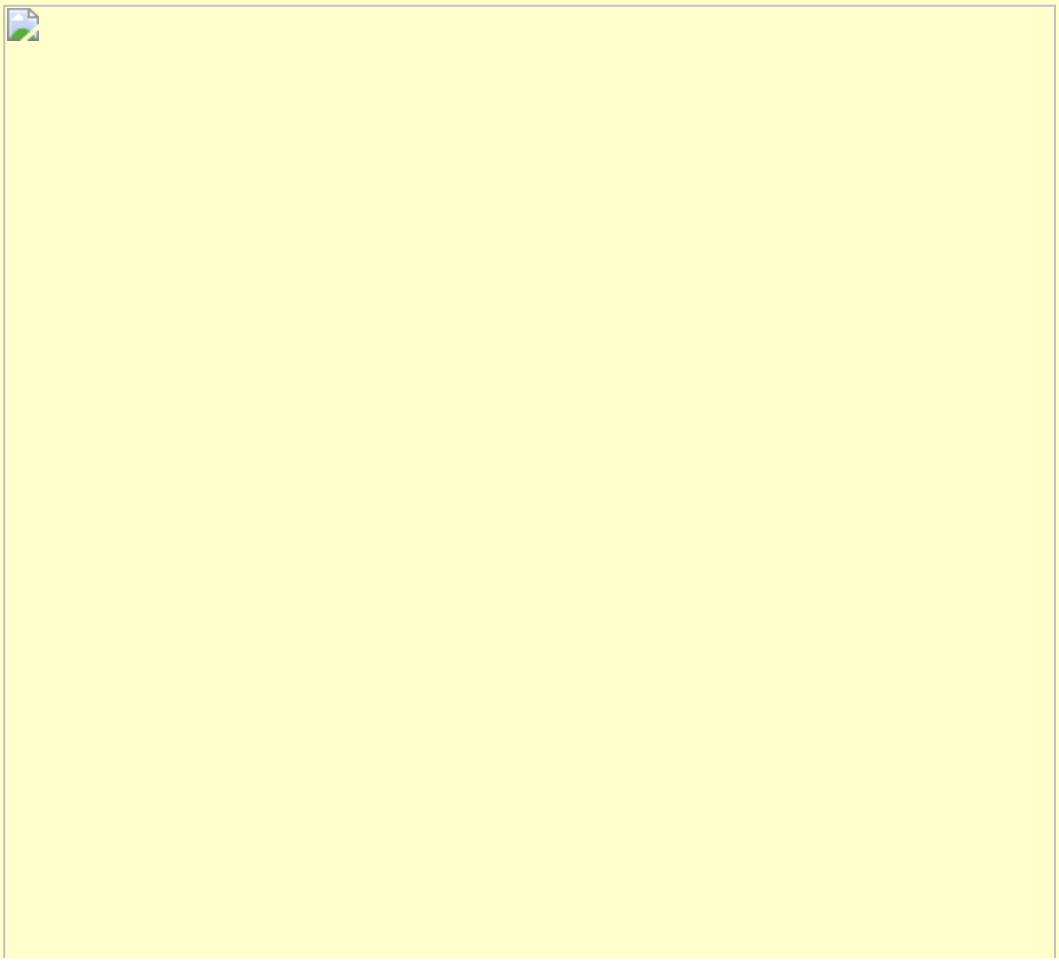


TOTAL HEAA CONTENT IN THIS SAMPLE IS 0.000% w/w

HYBRIDIZED *CORDYCEPS* SPECIES:

Plot 5 shows a sample of *Cordyceps sinensis* mycelium powder, grown in America on a solid substrate of grain, utilizing unique culture parameters and a specially hybridized, non-GMO strain of *Cordyceps sinensis*. This strain is detailed in section two of this paper as the most potent *Cordyceps* yet known, either from the wild or cultivated. See plot 5:

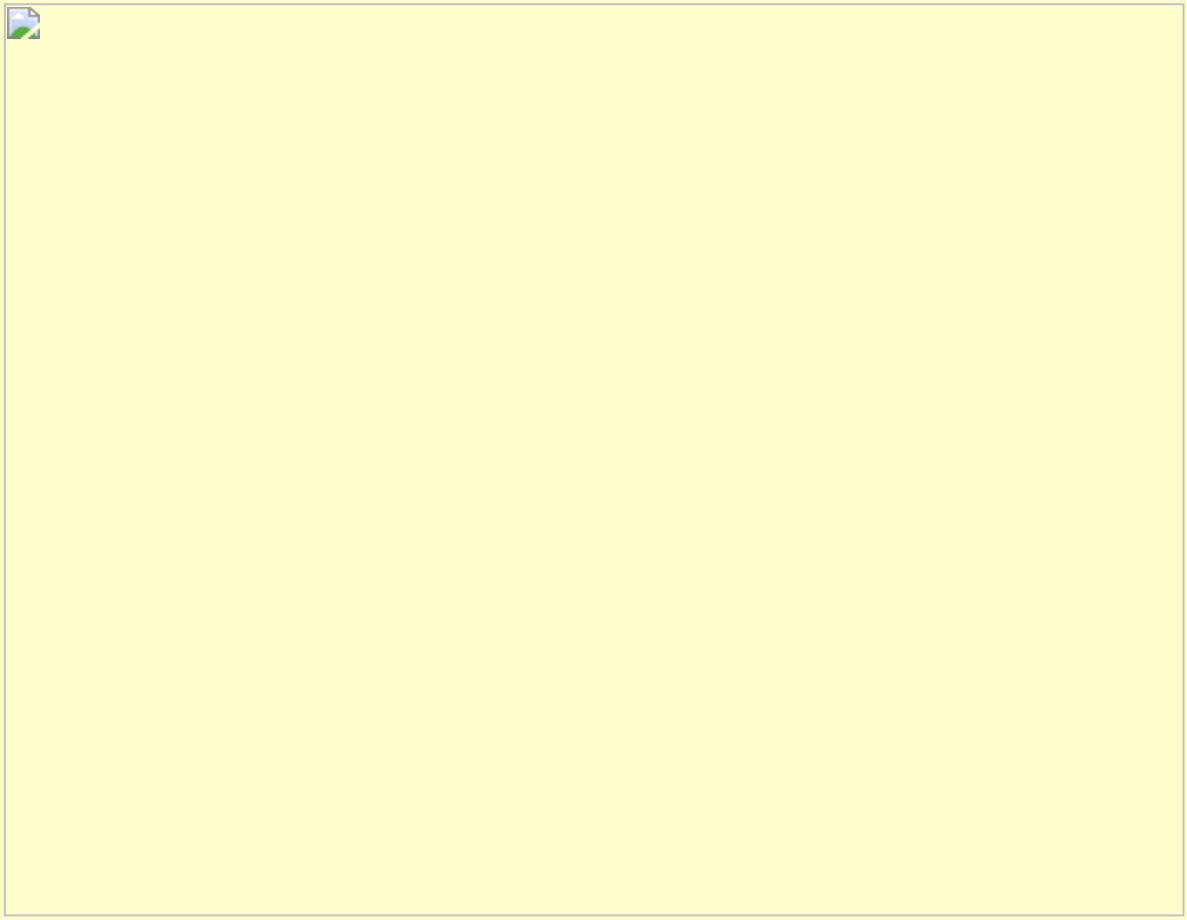
PLOT 5 – HYBRID *CORDYCEPS SINENSIS* ALOHAENSIS



TOTAL HEAA CONTENT IN THIS SAMPLE IS about .323% w/w

Plot 6 below is this same hybrid strain overlaid on a plot of wild *Cordyceps* to show the comparison in quantity of active ingredients as well as the qualitative similarities in the cultivated verses the wild *Cordyceps*. The secondary metabolites produced are very nearly identical in these two specimens of *Cordyceps*. See plot 6:

PLOT 6 – COMPARISON OF WILD TO HYBRID *CORDYCEPS*



PART II – HYBRIDIZATION METHODS AND CULTURE MODIFICATION

CULTURE METHODS AND SUBSTRATES:

In looking at the variations in quality from different strains and producers of *Cordyceps*, one must wonder what is it that causes this to be so. After all, a tomato is a tomato, no matter where it is grown. Yet with *Cordyceps*, even the same strain (CS-4) grown by different growers turns out to be entirely different from a standpoint of active ingredients. In looking into this question, it is first important to realize that there are two different methods used today in the cultivation of *Cordyceps*. There is the method primarily used in China, known as Liquid culture or Fermentation, in which the organism is introduced into a tank of sterilized liquid medium, which has been formulated to provide all of the necessary nutritional components for rapid growth of

the mycelium. After growth in the liquid medium, the mycelium is harvested by straining it out of the liquid broth and drying, after which it can be used as-is or further processed. Generally in this method the extra-cellular compounds, which were exuded by the fungus during the growth cycle, are discarded with the spent broth. This represents a major loss of bioactive compounds as many of the active ingredients are extra-cellular in nature, and are found only in small concentrations in the mycelium.

The second cultivation method is the solid-substrate method followed by most growers in Japan and America. In this cultivation system the mycelium is grown in plastic bags or glass jars full of sterilized medium, which is almost always some type of cereal grain. This grain is usually rice, wheat or rye although many different types of grain have been used. After some period of growth, the mycelium is harvested along with the residual grain. While this is an easily mastered and low capital investment cultivation technique, the down side of this method is that the grain content is usually greater than the mycelium content. In many cases, the solid-substrate grown mycelium we tested was greater than 80% residual grain. However, a bonus to this method is that the extra-cellular compounds are harvested along with the substrate and mycelium.

Cordycepin is an example of one of the compounds that is primarily extra-cellular in nature. Many tests have been done on cultured *Cordyceps* mycelium for the presence of Cordycepin. What is found by these tests is that in solid-substrate grown *Cordyceps*, there is usually Cordycepin present, and in liquid-cultured *Cordyceps*, usually none. The presence or absence of Cordycepin is dependent upon, among other factors, by which method the mycelium was grown and harvested.

We can see from this that the culture method itself has an effect on the quality of the resultant *Cordyceps* product. Beyond the methodology itself, the next most important factor in the production of particular secondary metabolites (or target medicinal compounds) is the nature and composition of the substrate itself. (Zhang, Zhao, Wu, Bai 1992) While it would seem that a substrate that favors rapid and strong growth of the mycelium would be an ideal substrate to use, this is not necessarily the case. Substrates are chosen on availability and price, or on historical usage or preference in handling. But rarely have they been chosen on the basis of the end compounds produced. In fact, the only way to determine whether the substrate being used is the best choice or not, is to compare the resultant product after growth on that particular substrate with some standard. If the end goal of production is Cordycepin or Didioxyadenosine (or some other specific compound) - as it is with some of the pharmaceutical companies - then the analysis is fairly straightforward. Just look for the amount of Cordycepin or Didioxyadenosine present and work around that. But life in the health supplement industry is rarely so simple. First we have to assume that we know what it is that we are looking for. Since natural products such as *Cordyceps* are chemically very complex, the truth is that we do not really know all of the components that are bioactively important.

With this realization in mind, we set out on a mission. To produce the best *Cordyceps* possible. What is the best? Since we did not know the answer to that question, we decided to try to copy the natural, wild collected *Cordyceps as closely as possible*. We attempted this by altering the substrate composition and analyzing the resultant mycelial product for known bioactive compounds. Then altering the substrate again…and again…and again. We did this through several hundred different substrates and through many thousands of kilograms of resultant product. What we found was that there was not any single method, strain or substrate we could use that would yield the results desired.

SUBSTRATE:

The substrate of choice for most Chinese growers is a liquid media based upon silkworm residue, with added carbohydrates and minerals. This seems a logical choice, since this mushroom is found in nature growing on insects. Dried silkworm bodies are the by-product of an existing industry and have little other use. Therefore they are readily available and cheap. This silkworm-based substrate seems to yield a relatively high quality product. The only problem with silkworm-residue based substrate is that in the United States, the FDA requirements are for mycelial products to be produced on a normally consumed human food source. Silkworms do not fit into that category. They are also not available as a raw material source to most of the worlds *Cordyceps* cultivators. The most usual substrate for Japanese and American growers is rice. It was determined in our trials that rice is not a suitable substrate for *Cordyceps* production if the target medicinal compounds are considered. Rice does not allow the full range of secondary metabolites to be expressed by the fungus, and rice grown *Cordyceps* has tested inferior in all of our analyses of active ingredient. There is rarely any appreciable amount of Adenosine or Cordycepin present in rice-grown *Cordyceps*. Furthermore, there are growth-stunting metabolites which build up in the substrate when *Cordyceps* is grown on rice, limiting the growth stage to only about 22-24 days, and allowing no more than about 40% of the rice to be converted into mycelial mass. This figure of 40% represents the high end of conversion, and is usually around 25-30%. This means that when *Cordyceps* is grown on rice then dried and powdered, the resultant product is actually about 60-75% rice flour.

Rye grain is another substrate often used for solid culture, and it yields a higher quality product than rice, as long as a source of vegetable oil as an amendment is added to the growth medium at the time of substrate makeup. The oil provides necessary nutrients, which the organism utilizes for bioactive compound production. Rye has other disadvantages though. The compounds in rye, which give it that characteristic rye smell and taste, are not broken down by the *Cordyceps* and they concentrate in the final product. This rye taste and smell overcomes the characteristic *Cordyceps* taste and smell, and even though the resultant product is of better quality than the rice grown mycelium, there are certain perceptual problems that needs to be overcome by the buyer to make this an economical alternative. Rice-grown *Cordyceps* may seem like a better product to

the average buyer because the rice does not mask the characteristic *Cordyceps* smell and taste. Most buyers in the health supplements industry tend to purchase bulk products on perception and faith rather than requiring an independent analysis. Rye also has growth-limiting factors, which causes the *Cordyceps* growth to stunt at about 28-30 days, although this can be overcome to a slight degree with the addition of about 1% ground oyster shell buffer to the medium at time of make-up. We tried many other sources of calcium, but they did not seem to work as well as the oyster shell calcium.

Millet is a very good choice of substrate when it is available. It has no strong taste or smell of it's own, it does not stunt the growth to any significant degree and it allows for the full expression of the secondary metabolites by the organism. It has another problem though, which is the high ratio of chitinous outer husk layer to starch. This outer husk is not broken down and represents a large portion of the final product weight, about 15%. The chitinous husk cannot be removed from the grain ahead of time, since doing so allows the grain to become too sticky during sterilization and a high degree of anaerobic contamination follows. The husk can be removed from the final product through mechanical means such as a time-of-flight separation, or the product can be used for hot water extractions or other processing. *Cordyceps* does not grow as fast on millet as it does on other grains, but the end product quality is higher.

White milo grain, also known as white kaffir corn or white sorghum is an excellent choice of substrate. The red variety of milo does not work nearly as well as the white variety as a substrate. White milo has all of the best characteristics; it is cheap, it has a high starch/husk ratio, it does not stunt the growth, it allows the full expression of bioactive compounds and has no strong odor or taste of its own to compete with the taste and smell of the resultant *Cordyceps* product. White milo when used alone however lacks some essential ingredients required for optimum growth by the *Cordyceps*. The addition of some portion of millet to the white milo speeds up the growth by a factor of 6 times. The millet to milo ratio is optimum at 1 part millet to 4 parts white milo.

Many farmers grow both white milo and the red milo in the same fields, or store them in the same silos, or otherwise do not keep the white and red separated. This is to be avoided when used as a *Cordyceps* substrate, since a small proportion of the red mixed in with the white can drastically reduce the growth rate and overall quality of the final product.

So from our substrate testing it was determined that the ideal medium for solid substrate growth of *Cordyceps* is as follows: 1 part white proso millet (husk on) to 4 parts of white milo (husk on), with the addition of 0.8% w/w of ground oyster shell and 1% w/w vegetable oil (peanut oil or soybean oil). Add water to equal 50% total moisture in the sterilized substrate. Precooking the grain mixture for 4-6 hours prior to sterilization tends to trigger a much faster growth response from the *Cordyceps*. On this medium, *Cordyceps* can be grown for long periods of time, allowing nearly complete conversion of the substrate to mycelium and the full expression of secondary

metabolites from the *Cordyceps*. The resultant *Cordyceps* when grown on this substrate is about 3-4% residual grain, or about 96-98% pure mycelium. The real benefit to this method of growing is the capture of the entire compliment of extra-cellular metabolites produced throughout the entire growth process. With the addition of certain growth triggering compounds to this mixture, *Cordyceps sinensis* is easily induced to fruit in culture without any insect material being present. However the formation of the fruitbody on this medium does not result in any significant change to the analytical chemistry profile.

CULTURE PARAMETER MODIFICATION: LOW TEMPERATURE HYPOXIA

Using the above-described substrate, the complete chemical profile of the cultivated C*ordyceps* still will not approach that of the wild collected *Cordyceps* unless it is grown under very specific conditions. *Cordyceps* sinensis produces a relatively large amount of free Adenosine when grown at normal atmospheric oxygen levels and room temperatures. It will also produce a large quantity of Uridine and Guanosine. But there is very little if any Cordycepin produced, and virtually no Hydroxyethyl Adenosine. For the organism to produce these compounds, it needs to be growth-stressed through the absence of oxygen, a drop in temperature and the total absence of light. Just growing it under cold and anaerobic conditions from the start will not do the trick, since when *Cordyceps* is grown under those conditions it forms a yeast-like anamorph that has a very different chemical profile. It must first be grown hot and fast, then tricked into converting its 'summertime' metabolites into the target medicinal compounds we are looking for. To get these target compounds, we found that we needed to follow a strict growth protocol: After inoculation on to the millet/milo substrate, the *Cordyceps* is grown at 20-22 degrees C, in diffuse light and at sea level atmospheric oxygen for 28-30 days. It is then moved into a specially controlled environmental chamber, where the oxygen is dropped to 50% atmospheric. The remainder of the growth atmosphere is made up of Nitrogen, Carbon monoxide and Carbon dioxide. The temperature is dropped to 3 degrees C, and all light is excluded. It is held under these conditions for about 15-20 weeks. This results in much of the Adenosine being converted to Cordycepin, Dideoxy-adenosine and Hydroxyethyl-adenosine. Many other unique nucleosides are also produced, with a final chemical profile identically matching that of the wild *Cordyceps* as can be clearly seen in Plot 6.

HYBRIDIZATION:

Once we had developed the substrate and growth parameters to optimize the target compounds, we started looking into the chemical profile differences from different strains of *Cordyceps sinensis*. Since there were so many strains of *Cordyceps*, and each strain has its own unique chemical profile, we tested all of the strains we were able to obtain. None of the known strains was shown to produce nearly the quantities of active ingredients found in the wild *Cordyceps*. So we started experimenting with ways to quantitatively increase the target compound production through the hybridization of *Cordyceps* strains; to cross breed them in order to gain greater production of target

compounds. This was quite a challenge. Since spore collection and separation is very time consuming and results in entirely too much unknown variations, we felt this method would take too much time before we had reliable results. Rather we took a novel approach. We experimented with various ways to get different strains of the fungi to perform their own nuclear fusion. There are several chemicals known to trigger this exchange of genetic material between unlike cells. Nicotinic acid for instance, can be used to create hybridized mycelium. This compound is difficult to use and yields unreliable results. After trying several different compounds to trigger this fusion, what we settled on was snake venom. See Illustration 1

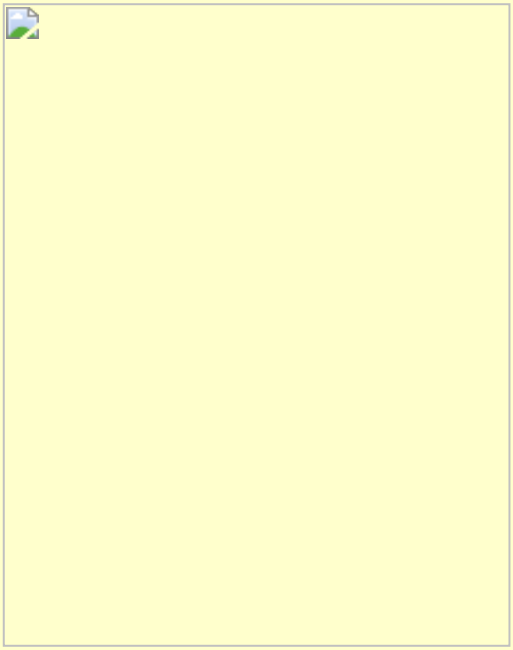


Illustration 1 – Collecting snake venom

SNAKE VENOM AS A HYBRIDIZATION AGENT:

We used purified snake venom from the Western Diamondback Rattlesnake (*Crotalus atrox* see illustration 2) [Sigma Scientific, St Louis Missouri, USA] for our hybridization techniques. The snake venom is added to the agar medium in quantities that alters the growth but does not prove toxic to the strain in question. This range of snake venom is from 10 mg to 30 mg per 300 ml of agar medium. The venom is not heat stable and must be added aseptically after sterilization of the medium. The agar used for this hybridization is an Aloha Medicinals Inc. proprietary agar named R7 Agar, consisting of malt extract, activated carbon, minerals and humus – the carbon-rich ash residue from a coal burning industrial process. For the exact recipe see table 4. Other agars could probably be used as well. This just happens to be our production agar that we use everyday, and once we found that it also worked with the snake venom for hybridization, we found no reason to experiment with any other agar.

TABLE 4 – R7 AGAR RECIPE

2.1 L	Distilled Water

50 g	Light Malt Extract
34 g	Agar
10 g	Humus
5 g	Activated carbon
1 g	$MgSO_4$
10 ml	1% KOH solution

ILLUSTRATION 2

***Crotalus atrox* - Western Diamondback Rattlesnake**

HYBRIDIZATION TECHNIQUE:

Petri dishes of this R7 agar medium are inoculated with mycelium from two different strains of the *Cordyceps* genus. These are usually two varieties of *C. sinensis*, although we have also crossbred *C. sinensis* with other *Cordyceps* species such as *C. militaris, C. sobolifera* and *C. ophioglosoides.* These different strains when inoculated together onto one petri dish will normally grow towards each other until they almost meet, at which point they form a zone of inhibition, where neither strain can grow. Eventually, one strain may prove stronger than the other and overgrow the plate, but they will remain genetically distinct; two different cultures residing in the same petri dish.

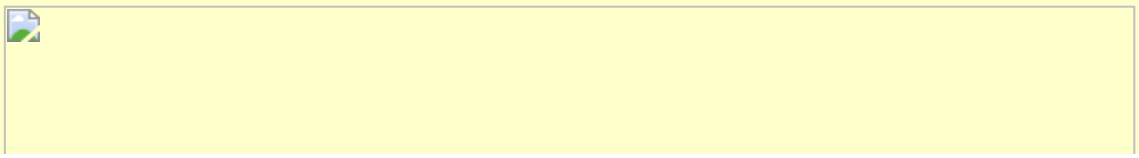
With the addition of a sufficient quantity of snake venom to the agar, we found that what happens is the two cultures grow towards each other until they meet and form their mutual zone of inhibition. This period of inhibition is short lived however, for in only about 2 or 3 hours the colonies each start sending out mycelial strands into this no-mans land, the zone of inhibition. These strands grow together and exchange nuclear material through their venom-weakened cell walls. They form a hybrid strain at this point of mutual contact. A new strain, one that is distinctly different from either of the parent strains. Within about 4 hours after first forming the zone of inhibition, the hybridization is complete and the colonies resume rapid growth towards each other. They become three colonies rather than the original two. There then exist in the same plate the original two colonies and a genetically distinct third…The Hybrid.

A section of the newly formed hybrid is carefully removed from the original zone of inhibition at the precise time that the colonies begin to fuse. That is during hour 3-4 after the initial meeting of the colonies. The hybrid is transferred to a new petri dish containing normal (non-snake venom) agar. Our quick method of determining hybridization is to inoculate a new dish containing normal agar with all three strains, the original two and the suspected hybrid. If the hybridization has in fact taken place, these are now three distinct colonies, and will form a mutual three-way zone of inhibition. If hybridization has failed to occur, then the suspected hybrid will readily fuse with either one or the other of the original colonies. This proves that our suspected hybrid is not genetically distinct from the original and we start anew. See Illustration 3

Once a hybrid is confirmed, it is tested for growth parameters. If it appears to be a vigorous and hardy grower on our substrate of choice, we grow out a quantity of mycelium, harvest it and analyze it for active ingredients. Through repeated testing in this way we were able to create the hybrid strain shown in Plot 6; a hybrid strain that is easily grown in solid substrate culture, with a potency greater than any other cultivated strain and at least equal in potency to the highest quality wild *Cordyceps*. We are referring to this new strain as *Cordyceps sinensis* Alohaensis. We are presently continuing this hybridization work with other species of *Cordyceps*, for the production of very specific target compounds. Top quality *Cordyceps* is no longer a health supplement only for the very rich. By utilizing these new methods of cultivation, the best quality *Cordyceps* is now within economic reach of even the common man throughout the world.

CONCLUSION:

There is a large and growing market worldwide for *Cordyceps* as a Medicine and as a Health Supplement. This large market demand coupled with the high prices of wild *Cordyceps* has given rise to an ever-increasing cultivation attempt by mushroom cultivators in many countries. Up until now, there has been no standard of quality, and no well-understood cultivation protocols with which to produce high quality *Cordyceps*. Compounding this has been the lack of any standardized test method by which to analyze and ascertain whether the cultivated Cordyceps was of a good quality or not. Here we have presented an overview of cultivation techniques, bioactive target compounds and quality index references, analytical protocols, substrate composition, cultivation methodology and hybridization techniques for production of the cultivars necessary in accomplishing these ends. This should serve as an outline for the production of high quality, economical *Cordyceps*, suitable for pharmaceutical and nutraceutical use, at a consistently high level of bioactive ingredients. This is a traditional medicinal substance with a long history of use, which is now ready to be used on an ever-increasing scale in the treatment of modern diseases.



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